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NTera 2 Cells: A Human Cell Line Which Displays Characteristics Expected of a Human Committed Neuronal Progenitor Cell

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We have identified a human cell line with a phenotype resembling committed CNS neuronal precursor cells. NTera 2/cl.D1 (NT2/D1) cells expressed nestin and vimentin, intermediate filament (IF) proteins expressed in neuroepithelial precursor cells, as well as MAP1b, a microtubule-associated protein (MAP) expressed in human neuroepithelium. NT2/D1 cells also expressed the cell adhesion molecules NCAM and N-cadherin which are thought to be important in cell–cell interactions within the neuroepithelium. These NT2/D1 cells also expressed small amounts of NF-L, α-internexin, NF-M, and MAP2c, indicating that they are committed to a neuronal fate. Previous studies have shown that, following RA treatment, a proportion of NT2/D1 cells terminally differentiate into neurons and that this occurs via an asymmetric stem cell mode of differentiation. In light of the identification of the neuroepithelial phenotype of NT2/D1 cells we decided to examine more closely the relationship of in vitro neurogenesis in NT2/D1 cells, during RA treatment to that of neurons in vivo. Three days after RA treatment, islands of NT2/D1 cells showed increased expression of neurofilament proteins and increased phosphorylation of NF-M. By 10–14 days, these cells began to resemble neurons morphologically, i.e., with rounded cell bodies and processes. These neuronal cells were clustered into clumps which rested on top of a layer of progenitor cells. In this upper layer, the neurons began to express MAP2b and tau and extinguished their expression of nestin. Recently, we developed a method for obtaining pure cultures of neurons from RA treated NT2/D1 cells. The phenotype of these postmitotic neurons is clearly dissociated from that of the untreated NT2/D1 cells. Given the data obtained in this study and the characterization of the neurons derived from NT2/D1 cells, we propose that NT2/D1 cells are a committed human neuronal precursor cell line which retains some stem cell characteristics and is capable only of terminal differentiation into neurons.

Key words: stem cells, neurofilament proteins, microtubule associated proteins, nestin, retinoic acid

INTRODUCTION

The mammalian central nervous system (CNS) is the culmination of a highly orchestrated, but poorly understood process, whereby $>10^{12}$ neuronal and nonneuronal cells are generated and assemble in a timely manner into complex multicellular networks. Although many aspects of this formidable developmental process have been elucidated, the cellular and molecular bases of CNS development are still largely unknown (see reviews by McConnell, 1991; McKay, 1989; Rakic, 1988; Temple, 1990). CNS neurons and macroglial cells are known to originate from a very small (relative to the total number of adult CNS neurons and glia) population of multipotential precursors located in the embryonic neuroepithelium that lines the ventricles and the neural tube. These precursor cells undergo a number of stem cell divisions which each yield one daughter cell that migrates and differentiates and another that retains its stem cell characteristics within the neuroepithelium (Lajtha, 1983; Rakic, 1988). During neurogenesis, numerous distinct classes of neurons and glia are "born" at specific times to facilitate the assembly of these cells into organized networks. The intrinsic and extrinsic mechanisms that govern the organization of the CNS remain largely unknown.

Lineage analysis of the CNS cell types produced by individual neuroectodermal progenitor cells indicate that they exhibit considerable regional heterogeneity (e.g., compare Leber et al., 1990 with Walsh and Cepko,

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1992). However, it is common for a single neuroectodermal precursor to generate several different types of neurons and glia (reviewed most recently in McConnell, 1991). Consistent with earlier anatomic data (Rakic, 1988), these cell lineage studies have shown that progenitor cells produce differentiated progeny at stereotypical times (Gray and Sanes, 1991). For example, neurons in the deeper cortical layers are born first followed by successively more superficial cortical neurons. Thus, while the progeny of a given precursor may consist of cells committed to different phenotypes, the individual daughter cells of multipotential precursor cells may be limited to phenotypes which are appropriate for that developmental time period. For example, the well studied O2A glial progenitor cell, which is found in the optic nerve and brain at specific time points in embryogenesis, is committed to become either astrocytes or oligodendrocytes yet is derived from the multipotential neuroepithelium (Raff, 1989). Heterochronic grafting experiments have shown that this process is regulated in part by external stimuli which affect either the precursor itself or direct the progeny of these precursors to assume a specific phenotype (McConnell, 1991). The progeny of precursor cells that become CNS neurons commit to this phenotype as they exit the cell cycle and begin to migrate away from the ventricular zone (McConnell and Kaznowski, 1991). These results imply that, while neurons and glia derive from multipotential precursor cells, they may progress through different stages in which their potential is more limited by environmental or intrinsic factors.

In the peripheral nervous system (PNS) there is strong evidence for the existence of stable precursor cells with restricted phenotypic repertoires. Recent lineage studies showed that individual neural crest cells are multipotent, capable of giving rise to any of the differentiated derivatives of the neural crest (Bronner-Fraser and Fraser, 1988). Neural crest-derived cells may proliferate further following migration, but they and their progeny are usually committed to a limited subset of phenotypes. This phenomenon is exemplified best by sympathoadrenal precursor cells which are capable of giving rise to sympathetic neurons, adrenal chromaffin cells and SIF cells (Doupe et al., 1985a,b; Anderson and Axel, 1986). Studies of these cells have been facilitated by the development of simple techniques for purifying multipotential neural crest cells and sympathoadrenal precursor cells for in vitro analyses. Indeed, it is possible to immortalize representatives of these precursor cell populations to generate multipotential cell lines and cell lines of restricted phenotype (Birren and Anderson, 1990; Lo et al., 1991; Murphy et al., 1991). Likewise, multipotential CNS neuroepithelial cells can be grown in culture (Cattaneo and McKay, 1990; Drago et al., 1991a,b; Gensburger et al., 1987; Murphy et al., 1990; Williams et al., 1991 and reviewed in Cattaneo and McKay, 1991; Reynolds and Weiss, 1992), and they have been subjected to limited analysis using either microculture (Temple, 1989; Reynolds and Weiss, 1992) or cell marking techniques (Cattaneo and McKay, 1990; Williams et al., 1991) which demonstrate that some of these cells generate only neuronal or glial progeny while others produce both. Despite these findings, immortalization of cultured neuroepithelial precursor cells has only yielded bipotential cell lines that give rise to neurons and glia (Bartlett et al., 1988; Fredericksen et al., 1988; Renfranz et al., 1991; Ryder et al., 1990; Snyder et al., 1992).

The availability of a cell line that is committed to differentiate into CNS neurons in response to a specific stimulus would enable detailed studies of the molecular basis of the complex process whereby cells acquire the phenotype of CNS neurons. NTera 2/clone D1 (NT2/D1) cells, a human teratocarcinoma-derived cell line, is a candidate for such studies since retinoic acid (RA) has been shown to induce these cells to terminally differentiate into postmitotic neurons (Pleasure et al., 1992). These neurons (referred to as NT2-N cells) closely resemble human CNS fetal neurons in that they express many cytoskeletal polypeptides, cell-surface antigens, and synaptic proteins typical of CNS neurons and they do not express any markers restricted to PNS neurons (Pleasure et al., 1992). When NT2/D1 cells are injected into nude mice the tumors contain differentiated neurons and a large component of residual cells which resemble the untreated cell line in culture (Andrews et al., 1984). Also, when treated with RA in culture, NT2/D1 cells produce differentiated neurons but retain a complement of "undifferentiated" cells that proliferate and resemble the untreated cells (Andrews, 1984; Lee and Andrews, 1986). Thus it seems that NT2/D1 cells are capable of going through a stem cell mode of division yielding daughter cells which retain their original phenotype and some cells which go on to terminally differentiate into neurons. These characteristics allow NT2/D1 cells to be classified as a stem cell line (Lajtha, 1983). In contrast to other teratocarcinoma cell lines which yield multiple cell types from multiple germ cell layers (Darmon et al., 1982; Jones-Villeneuve et al., 1982; Kuff and Fewell, 1980; Lang et al., 1989; McBurney, 1976; Paulin et al., 1982; Pfeifer et al., 1981; Strickland et al., 1980), NT2/D1 cells cannot be induced to yield derivatives other than neurons (Andrews et al., 1984; Andrews, 1983; Lee and Andrews, 1986). This unique feature of NT2/D1 cells led us to investigate whether the untreated NT2/D1 cells share any characteristics with neuroepithelial precursor cells. The results of these studies showed that untreated NT2/D1 cells have many molecular features of human neuroepithelial precursor cells but they

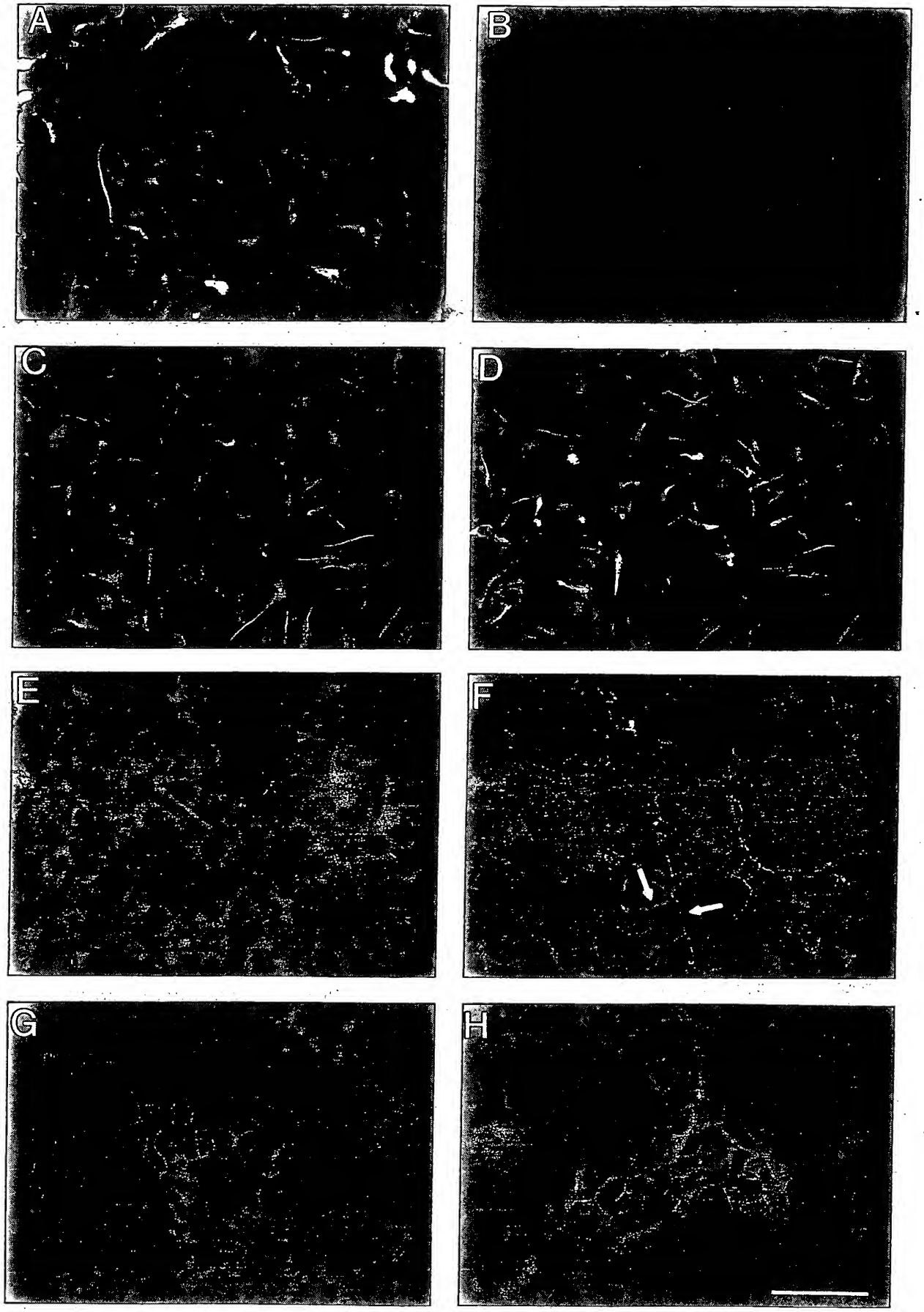


Fig. 1.

P-NF-M (Fig. 1D and Fig. 3B) as monitored at the mRNA level (for NF-L and NF-M) in northern blots and at the protein level (for all three) in western blots. These data differ from several earlier negative indirect immunofluorescence and immunoblot studies (Andrews et al., 1984; Andrews, 1984; Lee and Andrews, 1986). This may be explained by the increased sensitivity of recent modifications of these methods or the use of more sensitive mAbs in the present study. Indeed, low levels of NF-L and NF-M mRNA have been noted in NT2/D1$^-$ cells by some investigators (Abraham et al., 1991; Miller et al., 1990). The implication of these data, that the NT2/D1$^-$ cells might resemble neuronal progenitor cells, was supported by the detection of MAP2c mRNA (Fig. 3A) in NT2/D1$^-$ cells. MAP2c mRNA is an alternatively spliced form of the larger MAP2b mRNA (Papandrikopoulou et al., 1989), and the expression of MAP2c by embryonic cells is another early signal of commitment of neuroepithelial progeny to the neuronal lineage (Matus, 1988; Tucker, 1990). These observations were confirmed by conducting similar experiments on additional samples of NT2/D1 cells and related subclones, NT2/B9 and NT2/D3 (Andrews et al., 1984; Andrews, 1984), which we received fresh from Dr. P. Andrews. All of these cell lines also expressed the same markers detected in the NT2/D1 cells maintained in our laboratory.

Using indirect immunofluorescence in double-labeling studies, we showed that individual NT2/D1$^-$ cells coexpress nestin, vimentin, NF-L, α-internexin, NF-M, and keratins 8 and 18 (data not shown). In some of these cells, nestin and NF-M were grossly colocalized to the same filament network at the light microscopic level (Fig. 1C,D). We also noted that NF-M colocalized with NF-L, α-internexin, and vimentin (data not shown). This suggests that these proteins might coassemble into the same IFs. Other studies have noted the colocalization of neuronal IF proteins in the same network with vimentin in transfected nonneuronal cells (Chin and Liem, 1989; Monteiro and Cleveland, 1989; Pleasure et al., 1990). In the NT2/D1 cells, these different IF proteins were seen in a network of filaments in the perinuclear region that radiates into the cytoplasm and short processes of the NT2/D1$^-$ cells. In contrast, the keratin proteins were found in a different filamentous network suggesting that they do not coassemble with vimentin and the three neuron-specific IF proteins. These observations are similar to those made in other cell lines in which keratin and vimentin exist in different IF networks (McCormick et al., 1991). Normal human fetal cells do not appear to coexpress nestin and NF proteins in the same structures (Tohyama et al., 1992), but coexpression does occur in fetal rat cells (Fredericksen and McKay, 1988). Furthermore, in the developing chick, NF proteins are widely expressed in ventricular neuroepithelial cells (Bennett and DiLullo, 1985; Tapscott et al., 1981), and presumably these cells also express nestin. These findings suggest that the coexpression of nestin and NF proteins identifies embryonic cells in the initial stages of their commitment to become neurons in nonprimates. Several human primitive neuroectodermal tumor cell lines have been observed to coexpress nestin, vimentin, and NF proteins (Tohyama et al., 1992). These tumors may be comprised of neuronal progenitor cells that cannot exit the cell cycle and undergo terminal differentiation (Pleasure and Lee, unpublished observations). These cell lines express markers of neuronal committment and the stem cell state at the same time. NT2/D1$^-$ cells may be similar in many ways to these human cell lines, except that at least some proportion of the cells are capable of leaving the cell cycle following treatment with RA.

The prominent N-cadherin and NCAM immunoreactivity at cell–cell junctions (Fig. 1F,G,H) and the cobble stone staining pattern produced by the anti-N-cadherin mAb correspond to the normal localization of these proteins at cell–cell junctions (Volk and Geiger, 1986). The focal NCAM staining pattern of the NT2/D1$^-$ cells was not due to differential localization of the PSA forms of NCAM because antibodies against the PSA form and against the polypeptide backbone of NCAM showed identical distributions (Fig. 1G,H).

Taken together, these data suggest that NT2/D1 cells are a unique line of human neuroepithelial stem cells with a restricted ability to generate daughter cells committed to a neuronal phenotype.

The Molecular Phenotype of NT2/D1$^-$ Cells Is Clearly Dissociated From That of NT2/N Cells

To more sharply delineate the molecular phenotype of cycling neuronal progenitors, represented by NT2/D1$^-$ cells, we compared them to the terminally differentiated NT2-N fetal neurons derived from NT2/D1$^-$ cells following RA treatment. This was done by performing western and northern blot analyses to monitor the expression of several molecular markers of neuronal differentiation in both NT2/D1$^-$ cells and pure populations of NT2-N cells (Pleasure et al., 1992). Phase contrast photomicrographs of cells similar to those used for this analysis are presented in Figure 2. Figure 2 shows low power fields of NT2/D1$^-$ cells (Fig. 2A) and NT2-N cells (Fig. 2B) in >95% pure culture. NT2-N cell cultures appear similar to cultures of primary neurons. They extend numerous neuritic processes which cover the culture dish and maintain this morphology for long periods of time without any cell division (Pleasure et al., 1992). NT2-N cells also establish polarized processes which can be differentiated into axons or dendrites according to

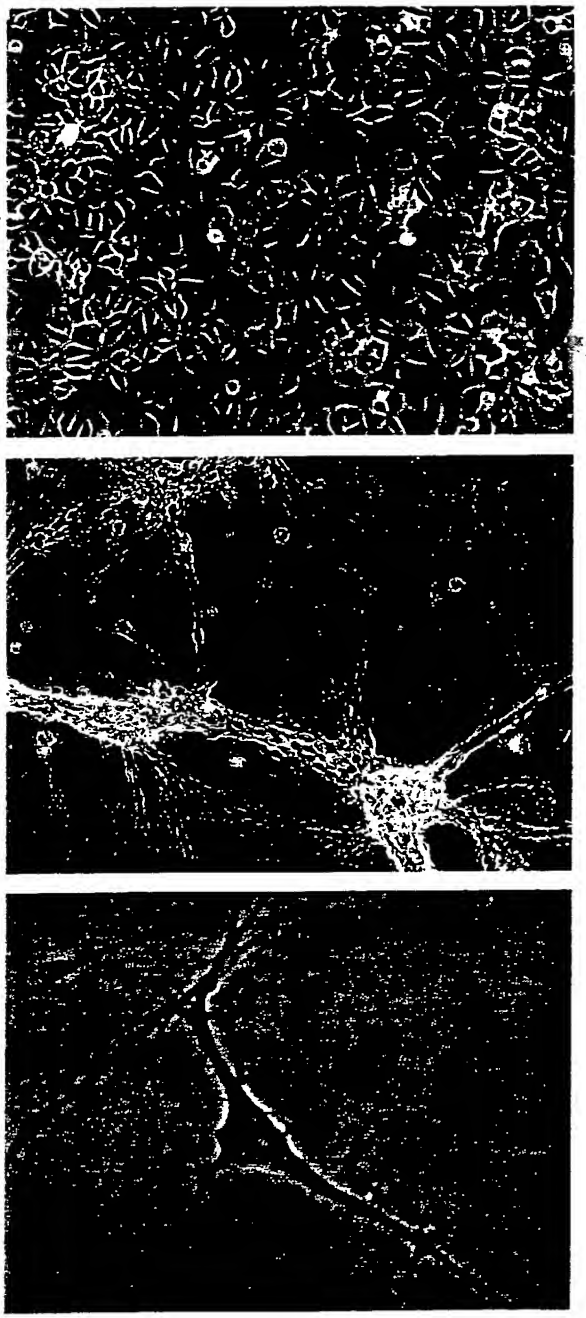


Fig. 2. Phase contrast photomicrographs of NT2/D1$^-$ cells and NT2-N cells. (A) Low power field of NT2/D1$^-$ cells; (B) low power field of NT2-N cells 4 weeks after purification; (C) a single NT2-N neuron with morphologically identifiable axons (arrowheads) and dendrites (arrows). The bar is 200 μm when applied to A and B and is 33 μm when applied to C.

morphologic or molecular criteria (Fig. 2C; Pleasure et al., 1992). Immunoblots conducted on NT2/D1$^-$ cell homogenates with the anti-nestin antiserum labeled a band >200 kDa consistent with the MW of human nestin (Tohyama et al., 1992), but this band was absent in extracts from pure cultures of NT2-N cells (Fig. 3B). Although a cDNA probe for nestin was unavailable, the polymerase chain reaction using RNA from NT2/D1$^-$ cells and NT2-N cells confirmed the presence of Nestin mRNA in NT2/D1$^-$ cells and its absence in NT2-N cells (data not shown). NF-L, NF-M, and α-internexin protein were all detected in immunoblots of cytoskeletal extracts from NT2/D1-cells (Fig. 3B) but they were less abundant than in similar extracts from the NT2-N cells. The NT2-N cells also contained more abundant NF-L and NF-M mRNAs than the NT2/D1$^-$ cells (Fig. 3A and data not shown). Probing the same northern blot with a probe for actin showed that the RNA loaded in each lane was approximately equal. MAP1b protein was far more abundant in the NT2-N cells than the NT2/D1$^-$ cells (Fig. 3B) but MAP1b mRNA levels were only mildly increased in NT2-N cells compared with NT2/D1$^-$ cells (Fig. 3A). It is apparent from examining both the northern and western blots that the increase in MAP1b protein during the differentiation of NT2-N cells from NT2/D1$^-$ cells far outweighs the moderate increase in the mRNA levels. This implies that MAP1b expression is at least partially regulated by translational control mechanisms.

In contrast, MAP2 expression was regulated by a different mechanism. For example, a probe from the 3′ end of the coding region of the MAP2 cDNA showed a single band at approximately 6.5 kb in the NT2/D1$^-$ cells while the same band was present in NT2-N cells (albeit at a much increased level) in addition to another band at about 9.0 kb. These two bands probably correspond to the previously described alternately spliced forms of MAP2, known as MAP2c and MAP2b (Papandrikopoulou et al., 1989). We confirmed this observation by probing the same northern blot using a probe prepared from the central region of the MAP2 cDNA which encodes the region spliced out of the MAP2c mRNA. In this experiment, there was no hybridizing band in the lane containing the NT2/D1$^-$ RNA, but the NT2-N cells only contained the 9.0-kb band. These results were confirmed at the protein level by immunoblotting with anti-MAP2 mAbs (Fig. 3B). In NT2/D1$^-$ cells no MAP2b (Fig. 3B) or MAP2c (not shown) protein was detectable, while in NT2-N cells both of these proteins were abundant (Fig. 3B and data not shown). This implies that NT2/D1$^-$ cells lack MAP2b, a marker of more mature neurons. Furthermore, they imply the existence of differential splicing events that are normally regulated in a developmental fashion which also can be regulated by RA induced terminal differentiation in NT2/D1 cells. Interestingly, our laboratory has found (Wertkin et al., submitted) that the β-amyloid precursor protein mRNA also is differentially spliced in RA treated NT2/D1 cells. These data support the notion that neurons have a distinct

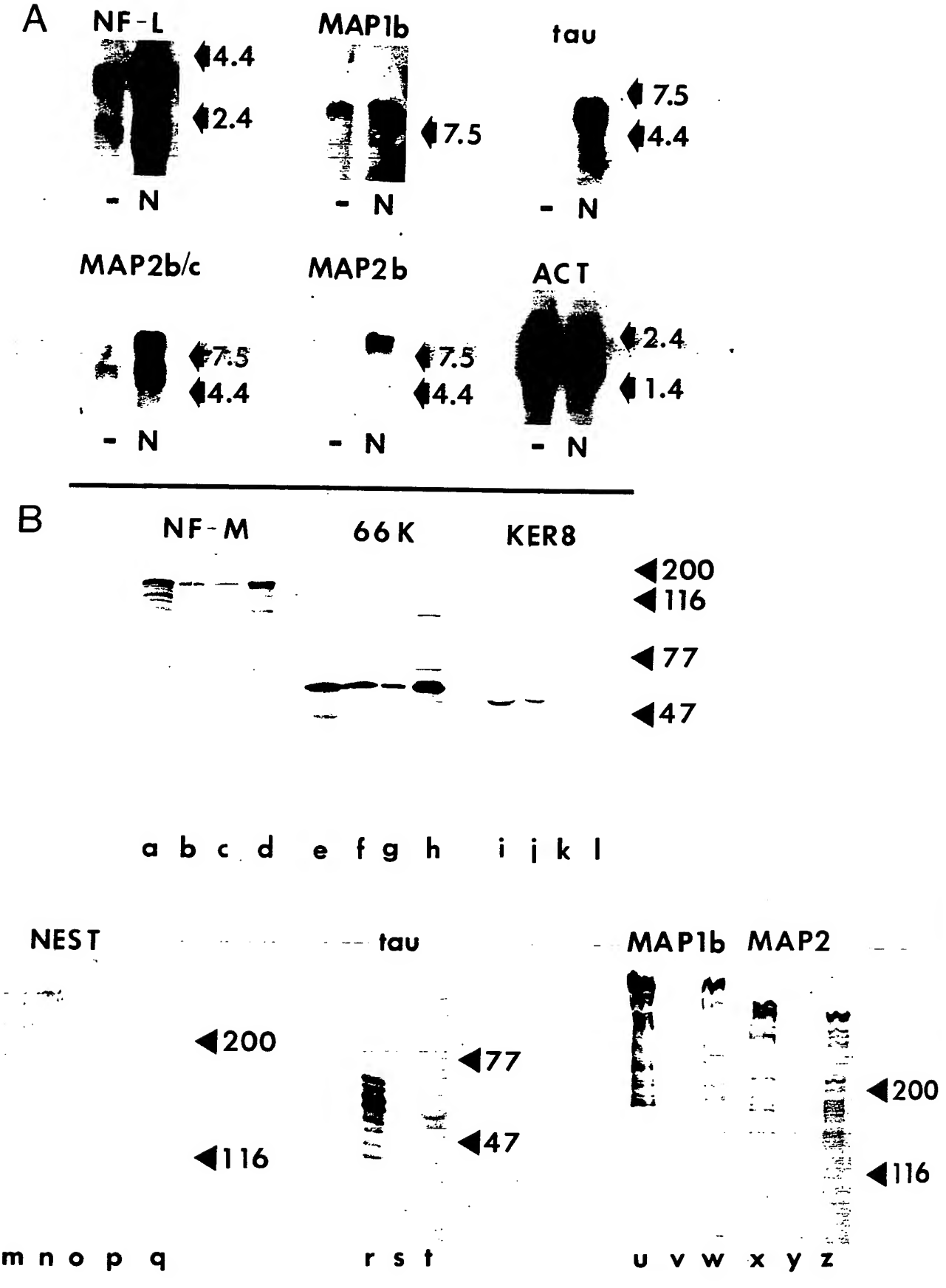


Fig. 3.

pattern of differential splicing for a wide variety of gene products (discussed in Li et al., 1989).

Based on the data described above, we conclude that the NT2/D1$^-$ cells express neuroepithelial markers and some of the earliest neuronal markers, but they do not express the full repertoire of markers found in differentiated neurons (such as MAP2b). NT2/D1$^-$ cells also do not express the high MW neuronal IF protein NF-H (not shown) which is normally expressed later in the developing CNS as well as in the terminally differentiated NT2-N cells (Carden et al., 1987; Julien et al., 1986; Pleasure et al., 1992; Tohyama et al., 1991). NT2/D1$^-$ cells also do not express two other MAPs normally found later in development (Matus, 1988), MAP1a (data not shown) and tau (Fig. 3A,B). Likewise, NT2/D1$^-$ cells do not exhibit the ultimate neurotransmitter phenotype of the NT2-N cells (Kleppner et al., 1991). Thus, the NT2/D1$^-$ cells exhibit a molecular phenotype distinct from that found in embryonic or mature neurons and we speculate that this phenotype might resemble that of a transient developmental population of just committed neuronal precursor cells.

Fig. 3. Molecular phenotype of NT2/D1$^-$ and NT2-N cells. (A) Northern blots examining the relative expression of a number of cytoskeletal mRNAs. The same blot, loaded with 2.5 μg of poly(A)$^+$ RNA per lane, was used for all the probes. The individual probes used for each panel are described in Materials and Methods. Lanes marked "−" contain RNA from NT2/D1$^-$ cells and lanes marked "N" contain RNA from NT2-N cells. The numbers to the right of each panel indicate RNA size markers in kb. The blots were exposed to X-ray film for varying times (NF-L = 3d; MAP1b = 10d; tau = 5d; MAP2b/c = 7d; MAP2b = 5d; ACT = 1d). (B) Western blots examining the expression of a number of cytoskeletal proteins. Each gel replica was probed with antibodies as described in Materials and Methods. Lanes a (near term human fetal cerebellum extract), b and c (100 and 25 μg of cytoskeletal extract from NT2/D1$^-$ cells, respectively) and d (25 μg of cytoskeletal extract from NT2-N cells) were probed with RMO254, an NF-M mAb. Lanes e (near term human fetal cerebellum extract), f, and g (100 and 25 μg of cytoskeletal extract from NT2/D1$^-$ cells, respectively), and h (25 μg of cytoskeletal extract from NT2-N cells) were probed with anti-NF66, an antiserum against α-internexin. Lanes i and j (350 and 50 μg of total extract from NT2/D1$^-$ cells, respectively) and k and l (50 and 150 μg of total extract from NT2-N cells, respectively) were probed with 35βH11, an anti-keratin 8 mAb. Lanes m (*E. coli* rat nestin fusion protein used as a positive control), n and o (100 and 25 μg of cytoskeletal extract from NT2/D1$^-$ cells, respectively), and p and q (25 and 100 μg of cytoskeletal extract from NT2-N cells, respectively) were probed with NS129, an anti-nestin antiserum. Lanes r (adult human tau) and s and t (50 μg of cytoskeletal extract from NT2/D1$^-$ cells and NT2-N cells, respectively) were probed with T14, a tau mAb. Lanes u (bovine MAPs) and v and w (50 μg of cytoskeletal extract from NT2/D1$^-$ cells and NT2-N cells, respectively) were probed with 1WM3G5, an MAP1b mAb. Lanes x (bovine MAPs) and y and z (50 μg of cytoskeletal extract from NT2/D1$^-$ cells and NT2-N cells, respectively) were probed with AP14, an MAP2 mAb. The panels labeled NF-M, 66K, and KER8 are all 10% SDS-PAGE gels. NEST is a 6% SDS-PAGE gel: tau is a 7.5% SDS-PAGE gel. MAP1b and MAP2 are 4–8% gradient SDS-PAGE gels.

Following RA Treatment NT2/D1 Cells Terminally Differentiate Into Neurons

Treatment with RA ultimately results in the production of terminally differentiated NT2-N cells which are postmitotic and express virtually all the markers of neurons. Since recent studies have described the molecular program which accompanies the differentiation of neuroepithelial cells into neurons and glial cells in the human CNS (Tohyama et al., 1991, 1992) we decided to compare the changes in NT2/D1 cell phenotype following RA treatment with the in vivo maturation of spinal cord neurons. Three days after treatment with RA, patches of cells (5–20) expressed markedly higher amounts of NF-L, NF-M, and α-internexin (Fig. 4a,b). Also, in the same patches of cells, highly phosphorylated (P+++) NF-M was expressed (Fig. 4d; and data not shown). Thus, within 3 days of RA treatment, the NT2/D1 cells showed (1) increased amounts of several neuronal IF proteins and (2) increased phosphorylation of NF-M. The patches of cells that showed increased NF-M expression had prominent perinuclear accumulations of NF-M as well as nestin (compare Fig. 4a,b). These perinuclear accumulations which contained NF-M and nestin (and also NF-L, α-internexin, and vimentin, data not shown) did not contain keratins 8 and 18 (Fig. 4c,d). This is further evidence that nestin coassembles into the same filamentous structures as vimentin and the NF proteins but not with the keratins. At 7 days the pattern was qualitatively similar but many more cells showed prominent NF-M, nestin and MAP1b staining (Fig. 5a,b,c).

During the first 10–14 days of RA treatment, the simple epithelial morphology of the cultures persisted despite the changes in NF and MAP1b expression. However, at around 14 days global changes in the overall architecture of the cultures became apparent. At this and later time points there remained a layer of flat epithelioid cells which continued to stain for nestin, keratin and vimentin and these cells appeared similar to the original untreated NT2/D1$^-$ cells. However, other cells began to pile up and form a second layer which was qualitatively different than the cells in the lower layer. For example, these cells extended long cytoplasmic processes from slightly rounded cell bodies (Fig. 6). Unlike the cells in the lower layer, the superficial cells no longer stained

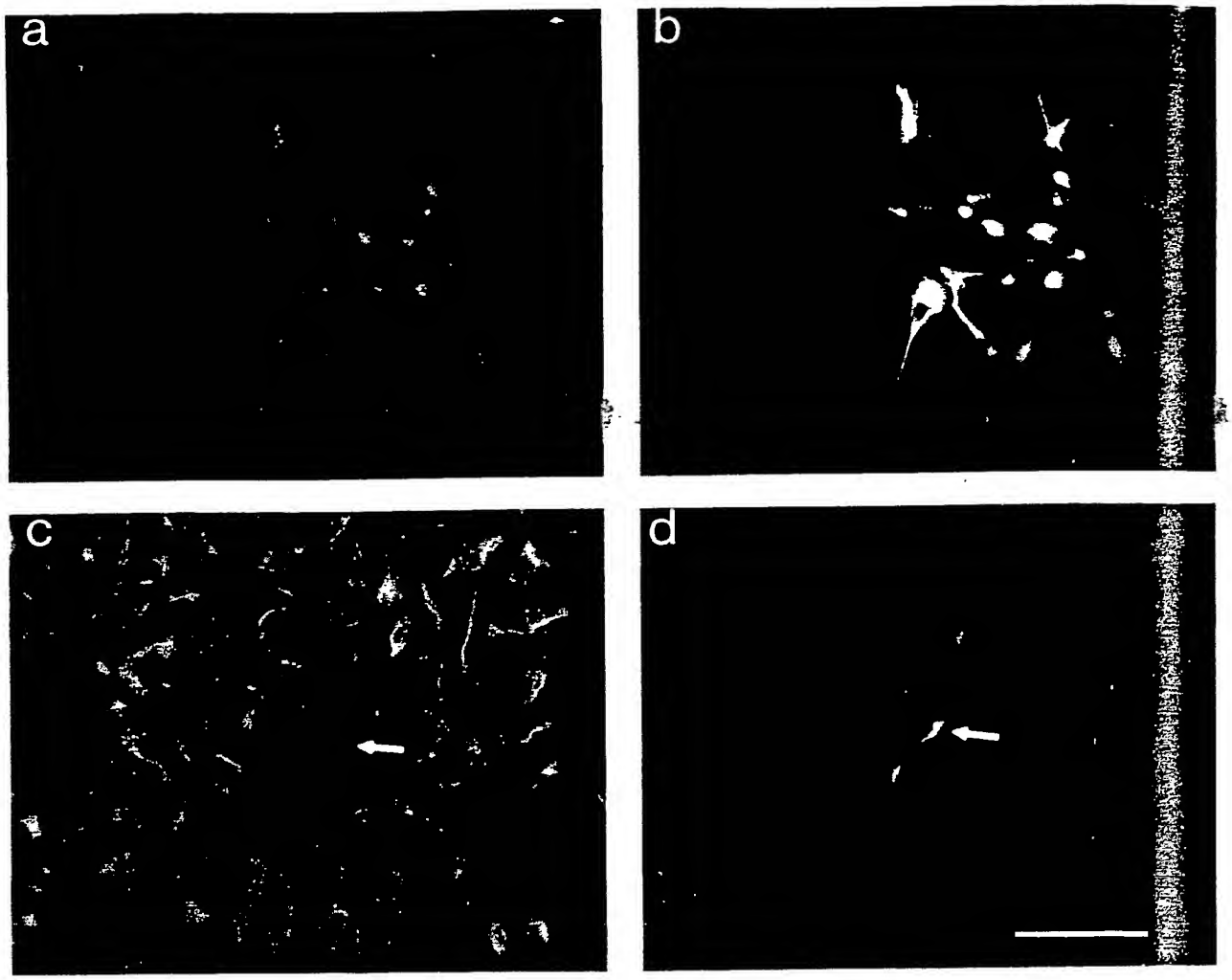


Fig. 4. NT2/D1 cells following three days of RA treatment: (a) rabbit antiserum to nestin and (b) the same field of cells stained with RMO254, a P(I) NF-M mAb; (c) Cam5.2 an mAb against keratins 8 and 18 and (d) the same field of cells with HO14, a rat P(+ + +) NF-M mAb. The bar is 50 μm.

with Abs to nestin, keratin, or vimentin (Fig. 6a,b) but continued to express P+ + + NF-M and the other neuronal IF proteins (Fig. 6c,d and Fig. 7a). These cells also expressed MAP2b (Fig. 7b) but still did not express tau or NF-H (data not shown).

The multilamination of these cultures was even more apparent by 3 to 4 weeks of RA treatment because of the extensive process outgrowth from the cells in the upper layers. Phase contrast microscopy of these cultures showed a dense multilayered mat of cells and immunofluorescence studies using anti-NF-M mAbs revealed a dense network of processes emanating from clumps of superficial cells (Fig. 6c,d and 7c). These cells and some of their processes stained intensely with MAP2b mAbs (Fig. 7d) and they exhibited the typical appearance of neurons in culture, i.e., rounded cell bodies and long processes. In another study, we showed that NT2-N cells elaborate polarized neurites which are distinguishable into axonal and somatodendritic domains by their expression of P(+ + +) NF-M (axons) or MAP2b (soma and dendrites) (Pleasure et al., 1992). This polarized morphology begins to become evident after 3–4 weeks of RA treatment (see arrows in Fig. 7c,d). Consistent with their identity as terminally differentiated neurons, the superficial cells also expressed tau and NF-H after three weeks of RA treatment (Fig. 7e,f,g,h, Fig. 8 and data not shown). Tau and NF-H appear relatively late during neurogenesis in the human spinal cord (Tohyama et al., 1991). The superficial cells in the upper layers of these mixed cultures appeared to be identical to the NT2-N cells grown in pure cultures (Pleasure et al., 1992).

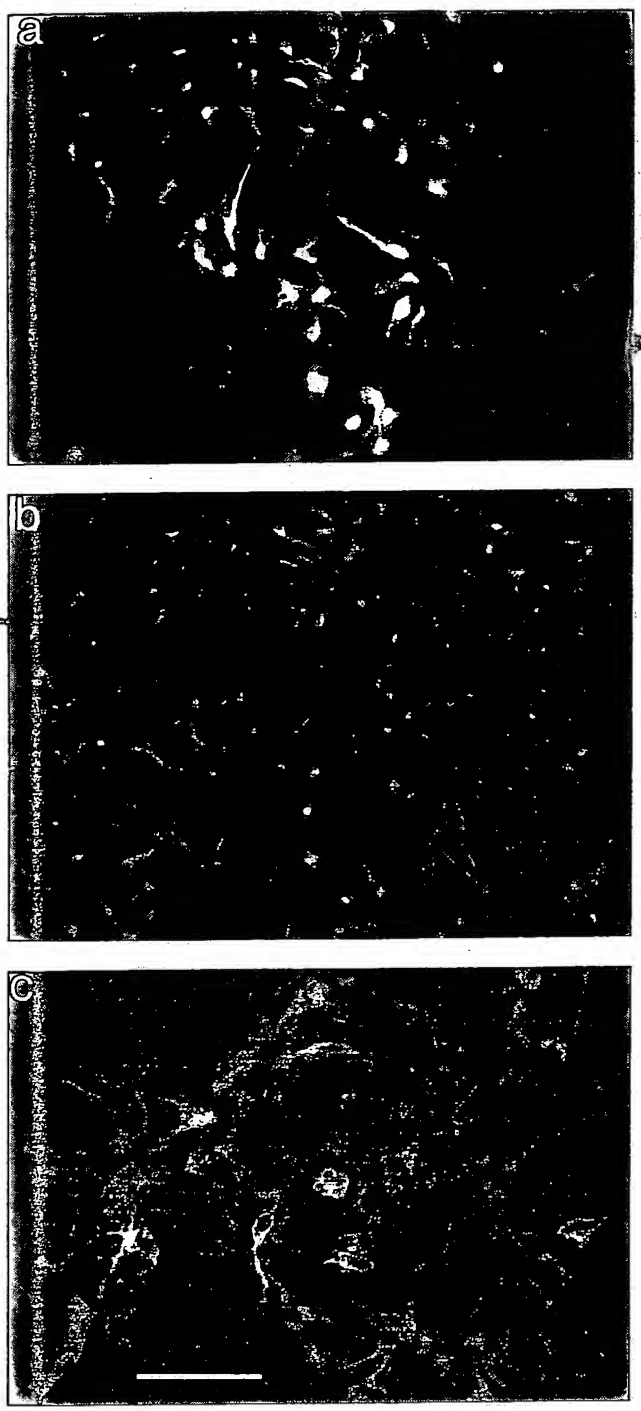


Fig. 5. NT2/D1 cells following seven days of RA treatment; (a) RMO254, a P(I) NF-M mAb and (b) the same field of cells with rabbit antiserum to nestin; (c) 1WM3G5, an MAP1b mAb. The bar is 50 μm.

A schematic illustration (Fig. 8) summarizes the immunocytochemical data described above. This figure shows the changes that occurred during RA treatment and applies to the most differentiated cells in the cultures at each time point. At each time point, the deepest layers in these cultures contained cells identical to the untreated NT2/D1$^-$ cells. This figure highlights three major molecular milestones in the RA induced neuronal differentiation of NT2/D1 cells which are shown explicitly in Figure 8B. First, after three days of RA treatment staining for neuronal IF proteins increased in intensity and P(+ + +) NF-M appeared. Second, after about 2 weeks of RA treatment, nestin staining was extinguished and MAP2 staining appeared for the first time in the developing neurons. Third, NF-H and tau (the quintessential markers of the terminal differentiation of neurons in vivo) appeared for the first time in cells treated for 3 weeks with RA.

DISCUSSION

NT2/D1 cells were derived initially by injecting the Tera 2 polyclonal cell line into nude mice and then cloning the resultant tumors (Andrews et al., 1984). This yielded a number of clonal cell lines three of which (NT2/D1, NT2/D3, and NT2/B9) have been characterized and shown to share a number of related characteristics (Andrews et al., 1984; Andrews, 1984). Several studies have examined the capacity of these cells to differentiate following treatment with RA (reviewed in Pleasure et al., 1992). Because neurons are the only differentiated cell type ever induced by RA treatment, we reasoned that NT2/D1 (and the other two clones) might represent neuronal progenitor cell lines. We addressed this issue in this study by examining the expression of various markers of neuroepithelial stem cells, neurons and glia in NT2/D1 cells.

The NT2/D1 cells were derived from a human teratocarcinoma; such tumors often contain differentiated neural elements including neurons and glial cells (Blaustein, 1977). Also, teratocarcinomas showing predominantly CNS neuroepithelial differentiation have been recognized as a genetic syndrome in mice (Caccamo et al., 1989a,b). Thus, the NT2/D1 cells (along with NT2/D3 and NT2/B9) may represent a clonal subpopulation of the original teratocarcinoma tumor that resemble immortalized neuronal precursor cells. This would explain the expression of NF proteins and MAP2c in these cells and the exclusive production of neurons following RA treatment. Notably, NT2/D1 cells also express cytokeratins 8 and 18, which is atypical of the human CNS neuroepithelium (Tohyama et al., 1991). However, these simple epithelial keratins are expressed in many carcinoma cell lines with an epithelial morphology (Miettinen et al., 1984) and may thus be a manifestation of the abnormal origin of these cells.

Since the untreated NT2/D1 cells have a phenotype

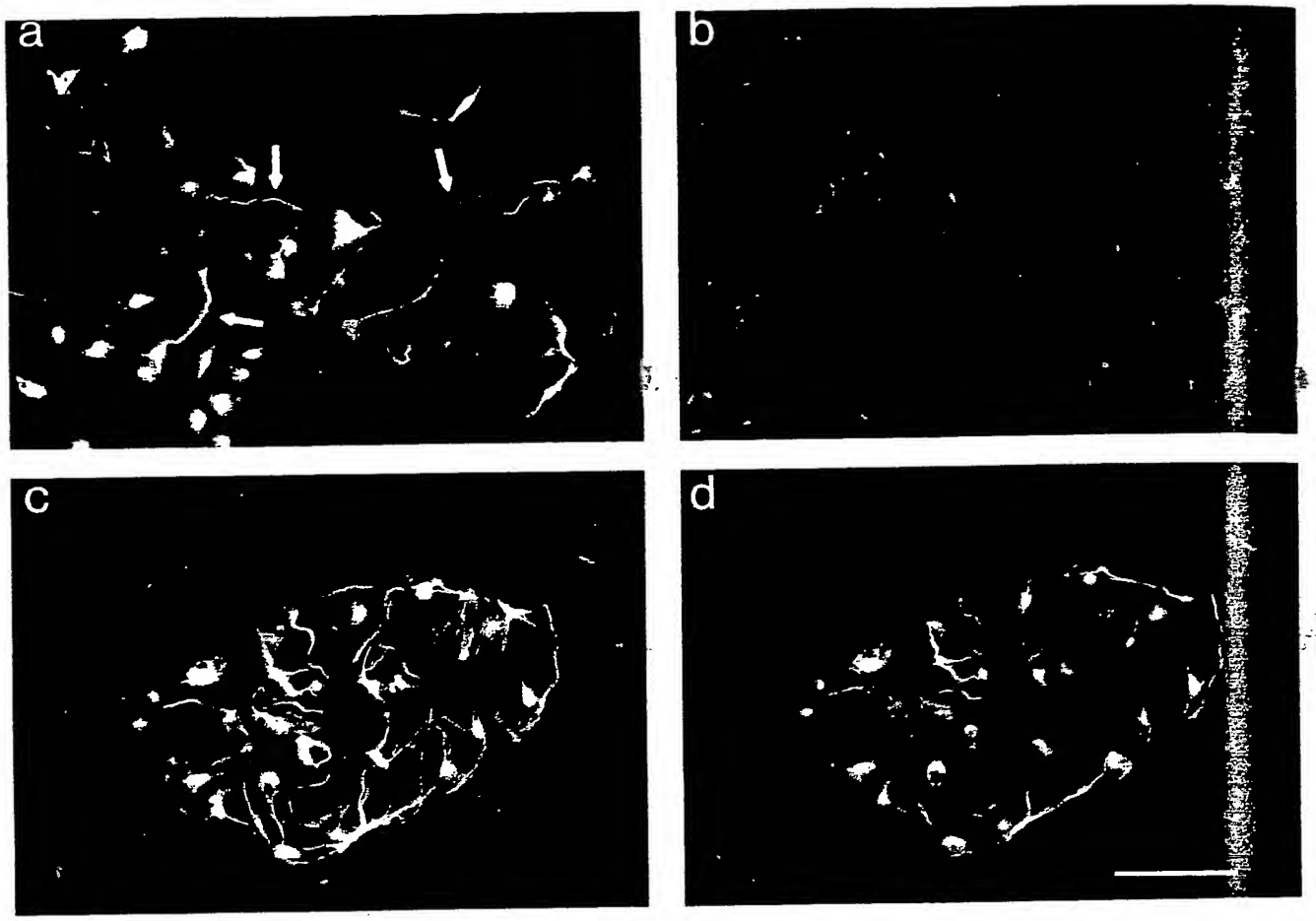


Fig. 6. NT2/D1 cells at the later stages of RA induced differentiation. (a) RMO254, a P(I) NF-M mAb and (b) the same field of cells with rabbit antiserum to nestin after 3 weeks of RA treatment; (c) RMO254, a P(I) NF-M mAb and (d) the same field of cells with rabbit anti-α-internexin after 4 weeks of RA treatment. The arrows indicate processes strongly stained with RMO254 but unstained with the nestin antiserum. The bar is 50 μm.

resembling in some ways committed neuronal precursor cells and RA treatment induces the progeny of NT2/D1 cells to adopt a stable neuronal phenotype reminiscent of human fetal CNS neurons (Pleasure et al., 1992), we sought to determine if RA-treated NT2/D1 cells progressed through a series of maturational steps similar to those observed recently in vivo in developing human spinal cord neurons (Tohyama et al., 1991, 1992). Remarkably, as summarized in the schematic shown in Figure 8, RA induced the sequential appearance or extinction of a number of neural markers in a programmatic manner that appeared to recapitulate maturational events in developing human spinal cord neurons.

Indeed, the fully mature neurons isolated from RA-treated NT2/D1 cells share many characteristics with normal neurons isolated from experimental animals (Pleasure et al., 1992). They do not divide, they maintain a stable neuronal phenotype over long periods of time, and are indistinguishable from terminally differentiated postmitotic neurons. This mode of differentiation is likely to be possible only in a stem cell tumor line, i.e., one in which a small number of the cells are responsible for the continued growth of the tumor while the majority

Fig. 7. Induction and accumulation of MAP2b and tau in RA-treated NT2/D1 cells. (a) and (b) NT2/D1 cells following 2 weeks of RA treatment stained with (a) HO14, a rat P(+++) NF-M mAb and (b) AP14, a mouse MAP2 mAb which does not react with MAP2c. The arrow indicates a cell positively stained with AP14. (c) and (d) show cells following 4 weeks of RA treatment stained with the same mAbs. The arrows indicate a cell more strongly stained with AP14 than HO14 and there are numerous processes stained with HO14 only. (e) and (f) NT2/D1 cells following 3 weeks of RA treatment stained with (e) rabbit anti-NF-L and (f) T14, a mouse mAb against tau. (g) and (h) show cells following 4 weeks of RA treatment stained with the same mAbs. The bar is 50 μm.

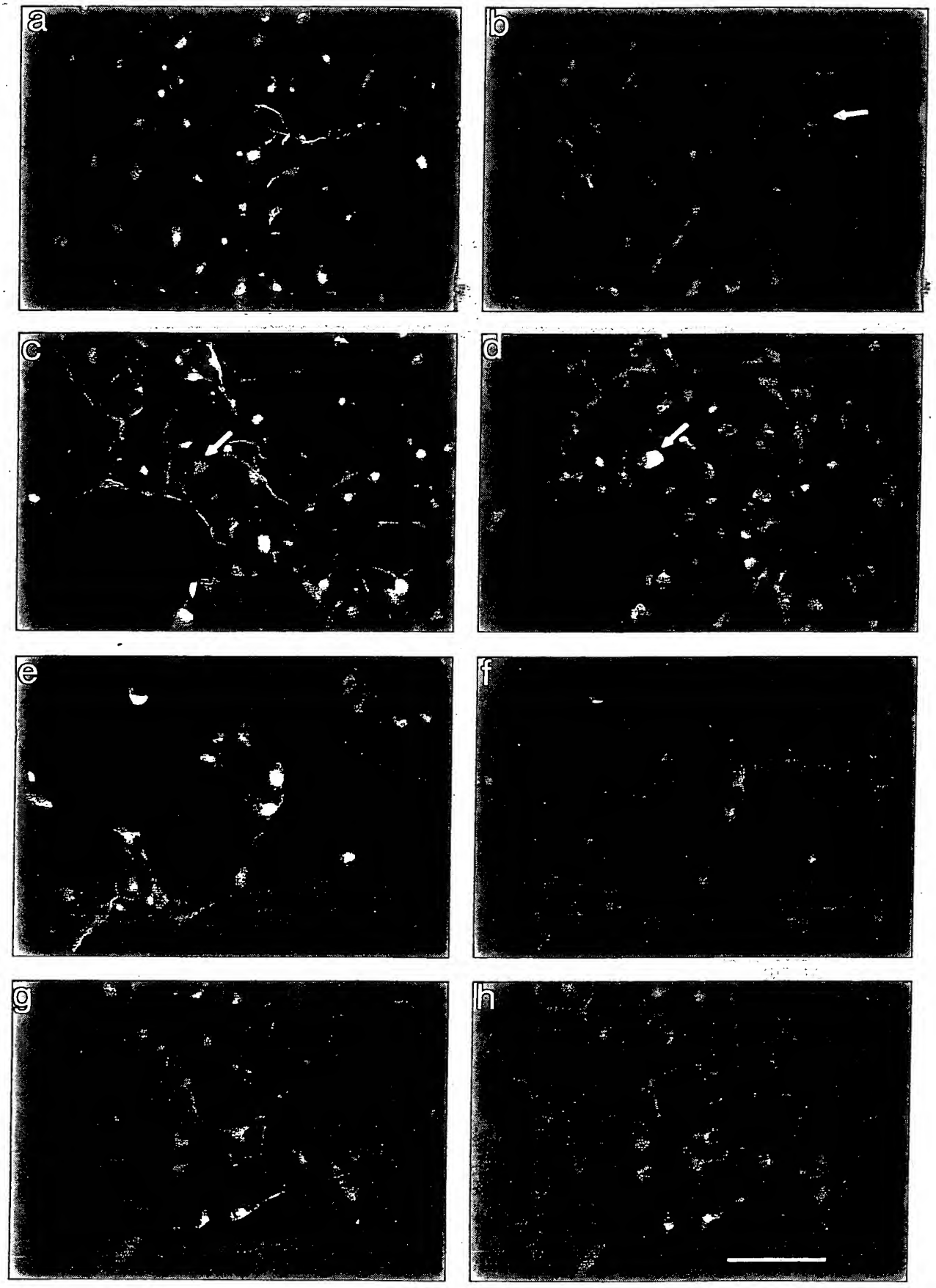
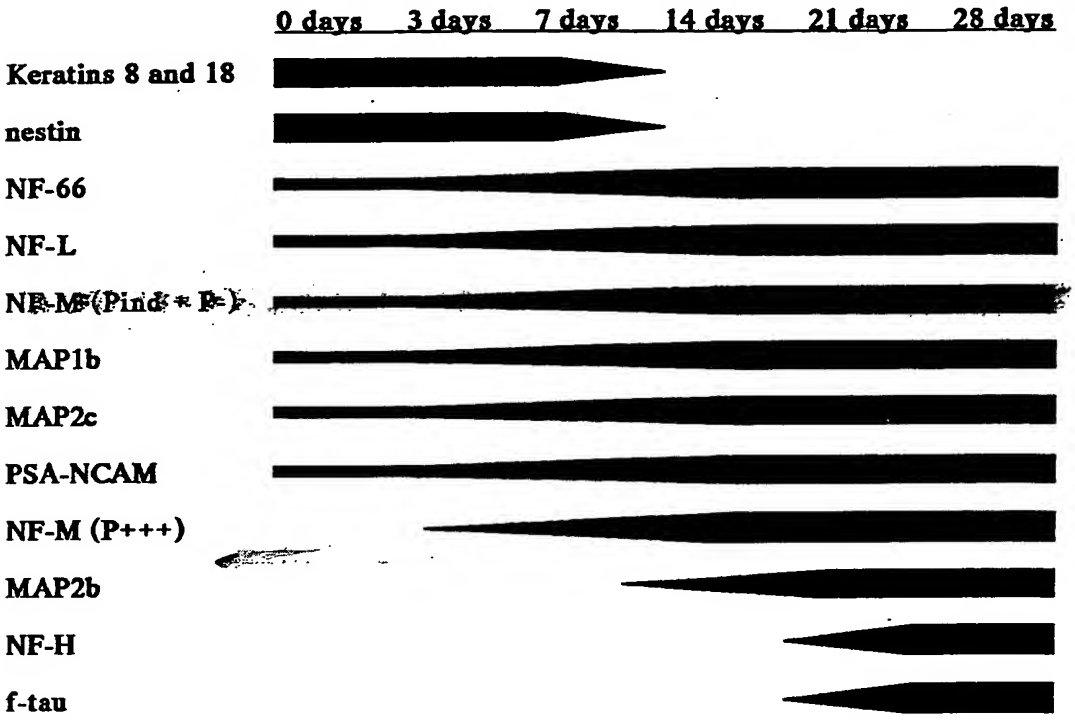


Fig. 7.

A. RA Treatment Induces Dramatic Changes in the Expression of Several Cytoskeletal Proteins.



B. Following RA Treatment NT2/D1 Cells Pass Through a Number of Intermediate States

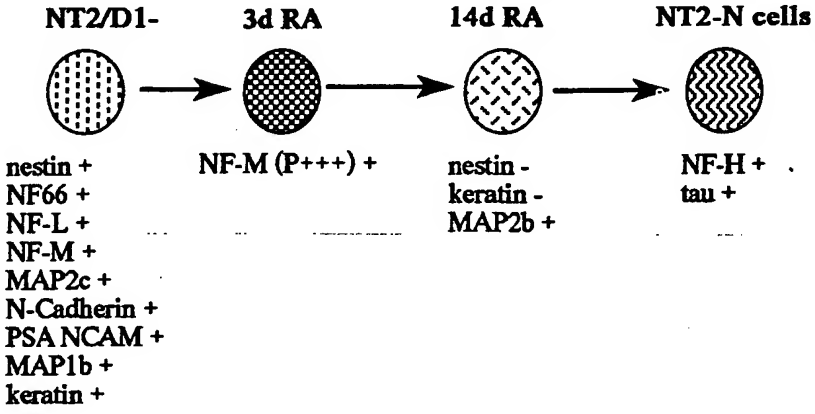


Fig. 8. (A) RA treatment induces dramatic changes in the expression of several cytoskeletal proteins. Summary of the changes in marker protein expression during the initial 4 weeks of RA treatment. Each of these markers was examined at the various time points by indirect immunofluorescence except for MAP2c where the increase was inferred from northern and western blots. The size of each bar does not indicate any relative differences between the expression of different cytoskeletal proteins. (B) Following RA treatment NT2/D1 cells pass through a number of intermediate states. The four distinct stages which NT2/D1 cells pass through during RA induced differentiation. At each time point global changes in the expression of several relevant markers are denoted by + or −.

of the cells (which make up the bulk of the tumor) are differentiated derivatives (Lajtha, 1983). In stem cell tumors, the primary neoplastic event may be abnormal persistence of a dividing stem cell phenotype in the face of normal regulating signals rather than, as in many other types of tumors, an event which causes quiescent cells to reenter the cell cycle (Lajtha, 1983). This type of mutation has been characterized in some stem cell leukemias which harbor a translocation mutation in a retinoic acid receptor (de The et al., 1991; Kakizuka et al., 1991). Addition of retinoic acid to these cells or treatment of patients harboring these tumors with retinoids causes these cells to terminally differentiate and the patients to enter a long-term state of remission (Warrell et al., 1991). Thus, this mutation alters the response of the tumor cells to a normal regulatory signal. While the mechanisms leading to the appearance of teratocarcinomas are enigmatic, the NT2/D1 cells appear to represent a population of early embryonic cells that has made the commitment to become neurons while retaining the capacity for cell division. The unlimited mitotic potential of NT2/D1 cells is likely to be a result of their transformation, but their ability to generate neuronal progeny appears to reflect a normal activity of neuronal progenitor cells in vivo.

Recent studies have shown that RA plays a major role during neuronal differentiation and specification. The region known as the floor plate, found ventrally in the developing spinal cord, has been shown to be capable of synthesizing RA (Wagner et al., 1990). Furthermore, cells in the developing spinal cord express nuclear receptors for RA, the cellular RA binding protein (CRABP) and the cellular retinol binding protein (CRBP) (Dencker et al., 1990; Maden et al., 1990, 1991). Thus, a potential source of RA and the mechanisms whereby cells can respond to RA are both present during CNS development. RA's teratogenic effects on the neural crest have been known for many years (Lammer et al., 1985), but recently it was shown that RA has pronounced effects on the development of the CNS if applied in pharmacologic doses during development. In RA treated xenopus embryos the rostral portion of the CNS (telencephalon, metencephalon, and myelencephalon) adopted a phenotype similar to that of more caudal regions of the CNS (the hindbrain and spinal cord) (Durston et al., 1989). Detailed analysis of this phenomenon showed that it was mediated by changes in the ultimate fate of more rostrally located neuronal cell types to resemble neuronal cell types found in caudal regions of the CNS (Ruiz i Altaba and Jessell, 1991). RA treatment of mouse embryos causes specific segmentation defects in the CNS and vertebrae along with altered patterns of Hox gene expression (Kessel and Gruss, 1991; Morriss-Kay et al., 1991). Thus, the effects of RA during CNS development emphasize a role for RA during important developmental periods when cell type and regional identity are specified.

This study describes a human cell line, derived from a human embryonal germ cell tumor, which displays properties expected of a human neuronal precursor cell line. While this cell line has an abnormal capacity for unlimited cell division, it retains the ability to respond to RA, an inducer likely to be important in the development of the human nervous system. Indeed, in response to RA some of the cells undergo irreversible differentiation into cells closely resembling neurons reflecting the development of neurons in vivo. Whether a population of cells similar to NT2/D1$^-$ cells exists in a stable form in the developing human CNS is unknown; it is possible that NT2/D1$^-$ cells resemble a transient phenotype among cells differentiating into neurons which has been immortalized by some as yet unknown insult. Many issues regarding this process in this cell line are yet to be understood. For instance, the fact that, despite the cell line's clonal origin, all the cells do not undergo synchronous differentiation may imply that some form of feedback inhibition on the differentiation process is likely to be operative, perhaps similar to that which probably occurs within clonal units developing in the neural tube (Rakic, 1988; McConnell, 1991). Nevertheless, the phenotype and characteristics of this cell line will make it valuable to investigators interested in studying RA's effects at the cellular and molecular level in the nervous system and others interested in studying and manipulating the development of neuronal precursor cells in vitro.

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REFERENCES

Abraham I, Sampson KE, Powers EA, Mayo JK, Ruff VA, Leach KL (1991): Increased PKA and PKC activities accompany neuronal differentiation of NT2/D1 cells. J Neurosci Res 28:29–39.

Anderson DJ, Axel R (1986): A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. Cell 47:1079–1090.

Andrews PW (1984): Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line *in vitro*. Dev Biol 103:285–293.

Andrews PW (1987): Human teratocarcinoma stem cells: Glycolipid antigen expression and modulation during differentiation. J Cell Biochem 35:321–332.

Andrews PW, Damjanov I, Simon D, Banting GS, Carlin C, Dracopoli NC, Fogh J (1984): Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Lab Invest 50:147–162.

Bartlett PF, Reid HH, Bailey K, Bernard O (1988): Immortalization of mouse neural precursor cells by the c-*myc* oncogene. Proc Natl Acad Sci USA 85:3255–3259.

Bennett GS, DiLullo C (1985): Transient expression of a neurofilament protein by replicating neuroepithelial cells of the embryonic chick brain. Dev Biol 107:107–127.

Birren SJ, Anderson DJ (1990): A v-*myc*-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. Neuron 4:189–201.

Blaustein A (1977): Germ cell tumors of the ovary. In "Pathology of the Female Genital Tract." New York: Springer-Verlag, pp 527–585.

Boisseau S, Nedelec J, Poirier V, Rougon G, Simonneau M (1991): Analysis of high PSA N-CAM expression during mammalian spinal cord and peripheral nervous system development. Development 112:69–82.

Bronner-Fraser M, Fraser S (1988): Cell lineage analysis shows multipotentiality of some avian neural crest cells. Nature (London) 335:161–164.

Caccamo DV, Katsetos CD, Herman MM, Frankfurter A, Collins VP, Rubinstein LJ (1989a): Immunohistochemistry of a spontaneous murine ovarian teratoma with neuroepitelial differentiation. Lab Invest 60:390–398.

Caccamo DV, Herman MM, Frankfurter A, Katsetos CD, Collins VP, Rubinstein LJ (1989b): An immunohistochemical study of neuropeptides and neuronal cytoskeletal proteins in the neuroepithelial component of a spontaneous murine ovarian teratoma. Am J Pathol 135:801–813.

Carden MJ, Trojanowski JQ, Schlaepfer WW, Lee VM-Y (1987): Two stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. J Neurosci 7:3489–3504.

Cattaneo E, McKay RDG (1990): Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature (London) 347:762–765.

Cattaneo E, McKay RDG (1991): Identifying and manipulating neuronal stem cells. TINS 14:338–340.

Chin SSM, Liem RKH (1989): Expression of rat neurofilament proteins NF-L and NF-M in transfected non-neuronal cells. Eur J Cell Biol 50:475–490.

Chiu F-C, Barnes EA, Das K, Haley J, Socolow P, Macaluso FP, Fant J (1989): Characterization of a novel 66 kd subunit of mammalian neurofilaments. Neuron 2:1435–1445.

Chuong CM, Edelman GM (1984): Alterations in neural cell adhesion molecules during development of different regions of the nervous system. J Neurosci 4:2354–2368.

Darmon M, Buc-Caron M-H, Paulin D, Jacob F (1982): Control by the extracellular environment of differentiation pathways in 1003 embryonal carcinoma cells: Study at the level of specific intermediate filaments. EMBO J 1:901–906.

de The H, Lavau C, Marchio A, Chomienne C, Degos L, DeJean A (1991): The PML-RARα fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell 66:675–684.

Dencker L, Annerwall E, Busch C, Eriksson U (1991): Localization of specific retinoid-binding sites and expression of cellular retinoic-acid-binding protein (CRABP) in the early mouse embryo. Development 110:343–352.

Doupe AJ, Patterson PH, Landis SC (1985a): Environmental influences in the development of neural crest derivatives: Glucocorticoids, growth factors and chromaffin cell plasticity. J Neurosci 5:2119–2142.

Doupe AJ, Patterson PH, Landis SC (1985b): Small intensely fluorescent cells in culture: Role of glucocorticoids and growth factors in their development and interconversions with other neural crest derivatives. J Neurosci 5:2143–2160.

Drago J, Nurcombe V, Bartlett PF (1991a): Laminin through its long arm E8 fragment promotes the proliferation and differentiation of murine neuroepithelial cells *in vitro*. Exp Cell Res 192:256–265.

Drago J, Murphy M, Carroll SM, Harvey RP, Bartlett PF (1991b): Fibroblast growth factor-mediated proliferation of central nervous system precursors depends on endogenous production of insulin-like growth factor I. Proc Natl Acad Sci USA 88:2199–2203.

Duband J-L, Volberg T, Sabanay I, Thiery JP, Geiger B (1988): Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. Development 103:325–344.

Durston AJ, Timmermans JPM, Hage WJ, Hendriks HFJ, de Vries NJ, Heideveld M, Nieuwkoop PD (1989): Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature (London) 340:140–144.

Fliegner KH, Wood TL, Pintar JE, Liem RKH (1990): Developmental expression of α-internexin, a novel neuronal intermediate filament protein examined by in situ hybridization. J Cell Biol 111:42a.

Fredericksen K, McKay RDG (1988): Proliferation and differentiation of rat neuroepithelial precursor cells *in vivo*. J Neurosci 8:1144–1151.

Fredericksen K, Jat PS, Valtz N, Levy D, McKay R (1988): Immortalization of precursor cells from the mammalian CNS. Neuron 1:439–448.

Geisert EE, Johnson HG, Binder LI (1990): Expression of microtubule-associated protein 2 by reactive astrocytes. Proc Natl Acad Sci USA 87:3967–3971.

Gensburger C, Labourdette G, Sensenbrenner M (1987): Brain basic fibroblat growth factor stimulates the proliferation of rat neuronal precursor cells *in vitro*. FEBS Lett 217:1–5.

Goedert M, Spillantini MG, Portier MC, Ulrich J, Crowther RA (1989): Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNA in human brain. EMBO J 8:393–399.

Gray GE, Sanes JR (1991): Migratory paths and phenotypic choices of clonally related cells in the avian optic tectum. Neuron 6:211–225.

Hatta K, Takagi S, Fujisawa H, Takeichi M (1987): Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. Dev Biol 120:215–227.

He X, Skapek SX, Wikstrand CJ, Friedman HS, Trojanowski JQ, Kemshead JT, Coakham HB, Bigner SH, Bigner DD (1989): Phenotypic analysis of four human medulloblastoma cell lines

also evidence an exclusive commitment to a neuronal phenotype.

MATERIALS AND METHODS

Cell Culture

NT2/D1 cells were cultured essentially as described previously (Andrews et al., 1984; Andrews, 1984; Pleasure, et al., 1992). NT2/D1$^-$ cells were passaged 1:3 twice per week in Opti-MEM with 5% FBS and penicillin/streptomycin. For experiments examining the consequences of RA treatment, NT2/D1 cells were seeded on coverslips (1×10^5 cells/well on a 24-well plate) and treated with 10 μM RA. For experiments requiring pure cultures of NT2-N cells, the cells were treated as described previously (Pleasure et al., 1992). Briefly, 2×10^6 cells were seeded in a 75-cm^2 flask and treated with 10 μM RA twice per week for 4 weeks. Following RA treatment the cells were replated at a reduced density. The following 2 days the cells were manually dislodged, counted and replated on Matrigel in medium containing mitotic inhibitors. RA was freshly prepared as a 10 mM stock solution in DMSO monthly. For experiments examining other NT2 subclones, freshly reconstituted NT2/B9 and NT2/D3 cells were kindly provided by Dr. Peter Andrews. These cells were immediately placed on coverslips and examined by indirect immunofluorescence. In this study we used 10 μM RA to induce differentiation in NT2/D1$^-$ cells. This concentration is surely superphysiologic, however, it was chosen for maximal effect; substantially similar qualitative effects occur at concentrations as low as 10 nM RA in these cells (Pleasure, et al., 1992).

Antibodies

The antibodies used for these experiments were either from our laboratory, provided by our colleagues, or purchased from a vendor. These included (1) rabbit anti-NF-L (Trojanowski et al., 1989a), (2) anti-NF-M and anti-NF-H mAbs (their names and characterizations are described in the text and the figure legends) (Lee et al., 1987), (3) rabbit anti-NF66 (NF66 and α-internexin are identical proteins) (Chiu et al., 1989), (4) rabbit anti-vimentin (Pleasure et al., 1990), (5) Cam 5.2, a mAb which sees keratins 8 and 18 (Becton Dickinson), and 35βH11, an anti-keratin 8 mAb (provided by Dr. Alan Gown), (6) AA6, an MAP1b mAb (Sigma) and 1WM3G5, another MPA1b mAb (Binder et al., unpublished), (7) T14, a tau mAb (Trojanowski et al., 1989b), (8) rabbit anti-NCAM and MenB, an mAb specific for PSA NCAM (Boisseau et al., 1991), (9) Moc1, another NCAM mAb (Molenaar et al., 1992), (10) GC4, an anti-N-cadherin mAb (Sigma), (11) AP14, an MAP2 mAb which does not see MAP2c (Geisert et al., 1990) and T34 an MAP2 mAb which binds MAP2b and c (Lee et al., unpublished), and (12) NS129, a rabbit antiserum raised to rat nestin expressed in *E. coli* (Tohyama et al., 1992).

Each NF-M and NF-H mAb is classified as described previously (Lee et al., 1987). Briefly, P(I) mAbs are those which react with their antigen regardless of phosphorylation state, P(−) mAbs are those which react with hypophosphorylated forms of NF-M or NF-H, and P(+) to P(+++) mAbs are those which react with increasingly highly phosphorylated forms of NF-M or NF-H.

Immunocytochemistry

Cells were washed with Opti-MEM without serum and fixed with 70% ethanol containing 0.15 M NaCl for 10 min at room temperature. The coverslips were overlayed with primary antibodies for 1 hr at room temperature and then washed four times with PBS for 1 hr. The coverslips were then overlayed with secondary antibodies for 1 hr at room temperature (donkey anti-mouse IgG coupled to Texas Red or TRITC, donkey anti-rat coupled to FITC, and donkey anti-rabbit coupled to FITC; Jackson Immunoresearch) and then washed four times in PBS for 1 hr before mounting in Aquamount (Lerner Labs).

Immunochemistry

For experiments examining cytoskeletal proteins by western blotting, MAP-enriched cytoskeletal samples were prepared by extracting the cells with microtubule assembly buffer containing taxol and Triton X-100 (0.1 M MES pH 6.8 containing 0.5 mM $MgSO_4$, 1 mM EGTA, 2 mM DTT, 2 mM GTP, 20 μM Taxol, 1% Triton X-100, and a cocktail of protease inhibitors) and centrifuging the samples at 30,000 rpm in a TL100 ultracentrifuge for 30 min. The pellets were solubilized in sample buffer without dye. For experiments examining cell adhesion proteins, total extracts were prepared by dissolving cells in sample buffer and boiling them for 15 min. The protein concentrations of the samples were determined using a Coomassie blue dye binding assay (Pierce). These samples were electrophoresed on SDS-PAGE gels and then electroblotted to nitrocellulose membranes for probing with antibodies using methods described previously in our laboratory (Lee et al., 1987).

Northern Blotting Analysis

Poly(A)RNA was prepared using a Micro Fast-Track kit (Invitrogen) according to the manufacturers instructions. RNA was separated on agarose/formaldehyde gels, blotted by capillary action to nylon membranes and crosslinked using a UV crosslinker. Northern blots were prehybridized and hybridized using Quikhyb solution according to the manufacturers instructions (Stratagene)

and probes were labeled by random priming (Prime-a-gene, Stratagene) using [α-^{32}P]DCTP as the labeled nucleotide. Blots were washed with 2× SSC + 0.1% SDS twice for 15 min at room temperature and then twice for 15 min at 55–60°C in 0.2× SSC + 0.1% SDS.

cDNA Probes

NF-L: The 1.3-kb *Eco*RI fragment was purified from plasmid NF5.1 (Myers et al., 1987).

NF-M: The 0.9-kb *Eco*RI fragment was purified from plasmid NF4 (Myers et al., 1987) or the 0.6-kb *Stu*I fragment was purified from plasmid pTZNF-M (Myers et al., 1987).

MAP1b: The 0.8-kb *Eco*RI fragment was purified from plasmid pSVMAP1b (Noble et al., 1989).

MAP2: The 1.3-kb *Xho*I *Eco*RI fragment was purified from plasmid pSVMAP2 (Lewis et al., 1988). The fragment is from the region encoding the COOH-terminal portion of MAP2 which is shared by MAP2b and MAP2c (Papandrikopoulou et al., 1989). The 1.3-kb *Bgl*II fragment was purified from plasmid pSVMAP2. This fragment is from the central region of the cDNA and corresponds to a region not found in MAP2c (Papandrikopoulou et al., 1989).

tau: The 0.8-kb *Bgl*I fragment was purified from plasmid pTau43 (Goedert et al., 1989).

RESULTS

Untreated NT2/D1 Cells Express Neuroepithelial and Neuroblast Markers

Previous characterization of NT2/D1$^-$ cells (in this paper, the untreated NT2/D1 cells are referred to as NT2/D1$^-$ cells) did not exhibit evidence of a differentiated phenotype. However, RA stimulates the production of differentiated daughter cells which resemble neurons (Andrews, 1984). While these neurons (which we term NT2-N cells) represented a minority of the total population of cells, NT2/D1 cells have not been shown to be capable of assuming a molecular phenotype consistent with any defined cell type other than neurons (Andrews et al., 1984; Andrews, 1984; Lee and Andrews, 1986; Pleasure et al., 1992). Here, we sought to examine the phenotype of NT2/D1 cells before treatment. Also, we wished to assess the extent to which RA treatment induced the progeny of NT2/D1 cells to express a well-defined group of polypeptides that define the molecular phenotype of neurons and that reflect the maturational state of cells during the development of cells of the neuronal lineage.

Nestin, a recently described intermediate filament (IF) protein is largely confined to neuroepithelial stem cells that coexpress vimentin, another IF protein (Frederickson and McKay, 1988; Lendahl et al., 1990; Tapscott et al., 1981; Tohyama et al., 1992). Similar to these stem cells, NT2/D1$^-$ cells expressed abundant nestin and vimentin. Both proteins were readily detected in immunoflurescence and immunoblotting experiments (see Fig. 1C and Fig. 3 for nestin and not shown for vimentin). Indirect immunofluorescence and northern blotting analysis of the NT2/D1 cells revealed the presence of MAP1b protein and mRNA, another molecular marker of neuroepithelial stem cells (Fig. 1E and Fig. 3). MAP1b (or MAP5) is a developmentally regulated high molecular weight (>280 kDa) microtubule-associated protein (MAP) (for reeent reviews of CNS MAPs, see Matus, 1988; Tucker, 1990) that is expressed in human germinal neuroepithelium (Tohyama et al., 1991). Furthermore, NT2/D1$^-$ cells also expressed polysialated (PSA) NCAM and N-cadherin on their cell surface (Figs. 1F,G,H). The relative abundance of these two cell adhesion proteins correlates inversely with the maturational state of neural cells in other systems and both appear very early in the neuroepithelium of the developing CNS (Chuong and Edelman, 1984; Duband et al., 1988; Hatta et al., 1987).

The first polypeptides to signal the commitment of cells to a neuronal phenotype are the middle molecular weight neurofilament (NF) protein, NF-M; and the two low molecular weight neurofilanent proteins, NF-L and α-internexin, although there are differences in the timing and sequence of their induction in telencephalic versus spinal cord neurons (Carden et al., 1987; Chiu et al., 1989; Fliegner et al., 1990; Julien et al., 1986; Kaplan et al., 1990; Tohyama et al., 1991). Notably, the progressive phosphorylation of NF-M reflects the increasing maturation of embryonic neurons and this developmentally regulated posttranslational modification of NF-M can be monitored closely using mAbs that recognize a cluster of phosphate-dpendent epitopes concentrated in the tail domain of this NF subunit (Carden et al., 1987; Tohyama et al., 1991). Since these neuron-specific IF proteins appear to signify the restriction of neuroepithelial derivatives to the neuronal lineage well before such cells acquire the distinct morphology of neurons, it was significant that NT2/D1$^-$ cells expressed low levels of NF-L (Fig. 3A), α-internexin (Fig. 1A and Fig. 3B), and

Fig. 1. Indirect immunofluorescent analysis of NT2/D1$^-$ cells shows that they express neuroepithelial and some neuronal markers: **(A)** rabbit antiserum to α-internexin and **(B)** the same field of cells with no primary antibody; **(C)** rabbit antiserum to nestin and **(D)** the same field of cells with RMO254, a P(I) NF-M mAb; **(E)** AA6, a MAP1b mAb; **(F)** GC4, an N-cadherin mAb; **(G)** MenB, a PSA NCAM mAb; **(H)** Moc1, and NCAM mAb. The arrows in F indicate cell–cell borders stained with GC4. The bar is 50 μm.

and transplantable xenografts. J Neuropathol Exp Neurol 48: 48–68.

Jones-Villeneuve EMV, McBurney MW, Rogers KA, Kalnins VI (1982): Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J Cell Biol 94:253–262.

Julien J-P, Meyer D, Flavell D, Hurst J, Grosveld F (1987): Cloning and developmental expression of the murine neurofilament gene family. Mol Brain Res 1:243–250.

Kakizuka A, Miller Jr WH, Umesono K, Warrell Jr RP, Frankel SR, Murty VVVS, Dmitrovsky E, Evans RM (1991): Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor, PML. Cell 66:663–674.

Kaplan MP, Chin SSM, Fliegner KH, Liem RKH (1990): α-Internexin, a novel neuronal intermediate filament protein, precedes the low molecular weight neurofilament protein (NF-L) in the developing rat brain. J Neurosci 10:2735–2748.

Kleppner SJ, Pleasure SJ, Lee VM-Y (1991): Neuronal cells derived from the cell line N-Tera 2 exhibit a cholinergic phenotype. Soc Neurosci Abstr 17:21.8.

Kuff EL, Fewell JW (1980): Induction of neural-like cells and acetylcholinesterase activity in cultures of F9 teratocarcinoma treated with retinoic acid and dibutyryl cyclic adenosine monophosphate. Dev Biol 77:103–115.

Lajtha LG (1983): Stem cell concepts. In Potten CS (ed): "Stem Cells: Their Identification and Classification." London: Churchill Livingstone, pp 1–11.

Lammer EJ, Chen DT, Hoar RM, Agnish ND, Benke PJ, Braun JT, Curry CJ, Fernhoff PM, Grix Jr. AW, Lott IT, Richard JM, Sun SC (1985): Retinoic acid embryopathy. N Engl J Med 313:837–841.

Lang E, Mazauric-Stucker M-L, Maelicke A (1989): States of developmental commitment of a mouse embryonal carcinoma cell line differentiating along a neural pathway. J Cell Biol 109: 2481–2493.

Leber SM, Breedlove SM, Sanes JR (1990): Lineage, arrangement, and death of clonally related motoneurons in chick spinal cord. J Neurosci 10:2451–2462.

Lee VM-Y, Andrews PW (1986): Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. J Neurosci 6:514–521.

Lee VM-Y, Carden MJ, Schlaepfer WW, Trojanowski JQ (1987): Monoclonal antibodies distinguish several differentially phosphorylated states of the two largest rat neurofilament subunits (NF-H and NF-M) and demonstrated their existence in the normal nervous system of adult rats. J Neurosci 7:3474–3488.

Lendahl U, Zimmerman LB, McKay RDG (1990): CNS stem cells express a new class of intermediate filament protein. Cell 60: 585–595.

Lewis SA, Wang D, Cowan NJ (1988): Microtubule-associated protein MAP2 shares a microtubule binding motif with tau protein. Nature (London) 242:936–939.

Li S, Klein ES, Russo AF, Simmons DM, Rosenfeld MG (1989): Isolation of cDNA clones encoding small nuclear ribonucleoparticle-associated proteins with different tissue specificities. Proc Natl Acad Sci USA 86:9778–9782.

Lo L-C, Birren SJ, Anderson DJ (1991): V-myc immortalization of early rat neural crest cells yields a clonal cell line which generates both glial and adrenergic progenitor cells. Dev Biol 145: 139–153.

Maden M, Ong DE, Chytil F (1990): Retinoid-binding protein distribution in the developing mammalian nervous system. Development 109:75–80.

Maden M, Hunt P, Eriksson U, Kuroiwa A, Krumlauf R, Summerbell D (1991): Retinoic acid-binding protein, rhombomeres and the neural crest. Development 111:35–44.

Matus A (1988b): Microtubule-associated proteins: Their potential role in determining neuronal morphology. Annu Rev Neurosci 11:29–44.

McBurney MW (1976): Clonal lines of teratocarcinoma cells *in vitro:* Differentiation and cytogenetic characteristics. J Cell Physiol 89:441–456.

McConnell SK (1991): The generation of neuronal diversity in the central nervous system. Annu Rev Neurosci 14:269–300.

McConnell SK, Kaznowski CE (1991): Cell cycle dependence of laminar determination in developing neocortex. Science 254:282–285.

McCormick MB, Coulombe PA, Fuchs E (1991): Sorting out IF networks: Consequences of domain swapping on IF recognition and assembly. J Cell Biol 113:1111–1124.

McKay RDG (1989): The origins of cellular diversity in the mammalian central nervous system. Cell 58:815–821.

Miettinen M, Lehto V-P, Virtanen I (1984): Antibodies to intermediate filament proteins in the diagnosis and classification of human tumors. Ultrastr Pathol 7:83–107.

Miller Jr WH, Moy D, Li A, Grippo JF, Dmitrovsky E (1990): Retinoic acid induces down-regulation of several growth factors and proto-oncogenes in a human embryonal cancer cell line. Oncogene 5:511–517.

Molenaar WM, de Leij L, Trojanowski JQ (1992): Neuroectodermal tumors of the peripheral and central nervous system share neuroendocrine N-CAM-related antigens with small cell lung carcinomas. Acta Neuopathol 83:46–54.

Monteiro MJ, Cleveland DW (1989): Expression of NF-L and NF-M in fibroblasts reveals coassembly of neurofilament and vimentin subunits. J Cell Biol 108:579–593.

Morriss-Kay GM, Murphy P, Hill RE, Davidson DR (1991): Effects of retinoic acid excess on expression of *Hox-2.9* and *Krox-20* and on morphological segmentation in the hindbrain of mouse embryos. EMBO J 10:2985–2995.

Murphy M, Drago J, Bartlett PF (1990): Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells *in vitro*. J Neurosci Res 25:463–475.

Murphy M, Bernard O, Reid K, Bartlett PF (1991): Cell lines derived from mouse neural crest are representative of cells at various stages of differentiation. J Neurobiol 22:522–535.

Myers MW, Lazzarini RA, Lee VM-Y, Schalepfer WW, Nelson DL (1987): The human mid-size neurofilament subunit: A repeated protein sequence and the relationship of its gene to the intermediate filament gene family. EMBO J 6:1617–1626.

Noble M, Lewis SA, Cowan NJ (1989): The microtubule binding domain of microtubule-associated protein MAP1B contains a repeated sequence motif unrelated to that of MAP2 and Tau. J Cell Biol 109:3367–3376.

Papandrikopoulou A, Doll T, Tucker RP, Garner CC, Matus A (1989): Embryonic MAP2 lacks the cross-linking sidearm sequences and dendritic targeting signal of adult MAP2. Nature (London) 340:650–652.

Paulin D, Jakob H, Jacob F, Weber K, Osborn M (1982): *In vitro* differentiation of mouse teratocarcinoma cells monitored by intermediate filament expression. Differentiation 22:90–99.

Pfeiffer SE, Jakob H, Mikoshiba K, Bubois P, Guenet JL, Nicolas J-F, Gaillard J, Chevance G, Jacob F (1981): Differentiation of a teratocarcinoma line: preferential development of cholinergic neurons. J Cell Biol 88:57–66.

Pleasure SJ, Lee VM-Y, Nelson DL (1990): Site-specific phosphorylation of the middle molecular weight human neurofilament protein in transfected non-neuronal cells. J Neurosci 10:2428–2437.

Pleasure SJ, Page CD, Lee VM-Y (1992): Pure, postmitotic polarized human neurons derived from NTera2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. J Neurosci 12:1802–1815.

Raff MC (1989): Glial cell diversification in the optic nerve. Science 243:1450–1455.

Rakic P (1988): Specification of cerebral cortical areas. Science 241: 170–176.

Renfranz PJ, Cunningham MG, McKay RDG (1991): Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. Cell 66: 713–729.

Reynolds BA, Weiss S (1992): Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.

Ruiz i Altaba A, Jessell TM (1991): Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos. Development 112:945–958.

Ryder EF, Snyder EY, Cepko CK (1990): Establishment and characterization of multipotent neural cell lines using retrovirus vector-mediated oncogene transfer. J Neurobiol 21:356–375.

Snyder EY, Deitcher DL, Walsh C, Arnold-Aldea S, Hartwieg EA, Cepko CL (1992): Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. Cell 68:33–51.

Strickland S, Smith KK, Marotti KR (1980): Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. Cell 21:347–355.

Tapscott SJ, Bennett GS, Holtzer H (1981): Neuronal precursor cells in the chick neural tube express neurofilament proteins. Nature (London) 292:836–838.

Temple S (1989): Division and differentiation of isolated CNS blast cells in microculture. Nature (London) 340:471–473.

Temple S (1990): Characteristics of cells that give rise to the central nervous system. J Cell Sci 97:213–218.

Tohyama T, Lee VM-Y, Rorke LB, Trojanowski JQ (1991): Molecular milestones that signal axonal maturation and the commitment of human spinal cord precursor cells to the neuronal or glial phenotype in development. J Comp Neurol 310:285–299.

Tohyama T, Lee VM-Y, Rorke LB, Marvin M, McKay RDG, Trojanowski JQ (1992): Nestin expression in embryonic human neuroepithelium and in human neuroepithelial tumor cells. Lab Invest 66:303–313.

Trojanowski JQ, Kelsten ML, Lee VM-Y (1989a): Phosphate-dependent and independent neurofilament protein epitopes are expressed throughout the cell cycle in human medulloblastoma (D283 MED) cells. Am J Pathol 135:747–758.

Trojanowski JQ, Schuck T, Schmidt ML, Lee VM-Y (1989b): Distribution of tau proteins in the normal human central and peripheral nervous system. J Histochem Cytochem 37:209–215.

Tucker RP (1990): The roles of microtubule-associated proteins in brain morphogenesis: A review. Brain Res Rev 15:101–120.

Volk T, Geiger B (1986): A-CAM: a 135-kD receptor of intercellular adherens junctions. I. Immunoelectron microscopic localization and biochemical studies. J Cell Biol 103:1441–1450.

Wagner M, Thaller C, Jessell T, Eichele G (1990): Polarizing activity and retinoid synthesis in the floor plate of the neural tube. Nature (London) 345:819–822.

Walsh C, Cepko CL (1988): Clonally related cortical cells show several migration patterns. Science 241:1342–1345.

Warrell RP Jr, Frankel SR, Miller WH, Scheinberg DA, Itri LM, Hittelman WN, Vyas R, Andreeff M, Tafuri A, Jakubowski A, Gabrilove J, Gordon MS, Dmitrovsky E (1991): Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-*trans*-retinoic acid). N Engl J Med 324:1385–1393.

Williams BP, Read J, Price J (1991): The generation of neurons and oligodendrocytes from a common precursor cell. Neuron 7:685–693.